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# Neither Dectin-2 nor the Mannose Receptor Is Required for Resistance to *Coccidioides immitis* in Mice

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**We investigated the roles of the mannose receptor (MR) and Dectin-2 in resistance to pulmonary coccidioidomycosis in C57BL/6 (B6) mice and in the interaction of myeloid cells with spherules, using B6 mice with targeted mutations in *Mrc1* and *Clec4n*. Spherules are the tissue form of *Coccidioides*, and we determined that the MR on bone marrow-derived dendritic cells (BMDC) was important for recognition of spherules (formalin-killed spherules [FKS]) and for secretion of interleukin 10 (IL-10) and proinflammatory cytokines in response to FKS by both elicited macrophages and BMDC. Infected MR knockout (KO) mice produced more IL-10 in their lungs than did B6 mice, and MR KO mice also made more protective Th-17 cytokines. In contrast to the MR, Dectin-2 was not required for recognition of FKS by BMDC or for the production of cytokines by BMDC in response to FKS. However, Dectin-2 KO was required for stimulation of elicited peritoneal macrophages. Despite that, lung cytokine levels were not significantly different in Dectin-2 KO mice and B6 mice 14 days after infection, except for IL-1β, which was higher in Dectin-2 KO lungs. Although both Dectin-2$^{-/-}$ and MR$^{-/-}$ myeloid cells had reduced proinflammatory cytokine responses to FKS *in vitro*, neither MR nor Dectin-2 deficiency reduced the resistance of B6 mice to pulmonary coccidioidomycosis.**

Coccidioidomycosis is a fungal infection endemic to the Western Hemisphere that is caused by either of two closely related species, *Coccidioides immitis* or *C. posadasii*. Both are dimorphic fungi that grow as spore-forming molds in dry, alkaline desert soil and cause infections in vertebrates after the spores are inhaled. In a vertebrate host, spores (arthroconidia) convert to spherules, a unique and pathognomonic structure (1). When spherules are mature, they are multicellular structures that contain hundreds of endospores that are released when spherules rupture. In turn, endospores can grow into spherules, repeating the cycle. Endospores and small spherules can be ingested by host phagocytes, but mature spherules grow to be 100 to 150 μm in diameter, too large to be ingested by phagocytic cells. In pathological sections, spherules are often extracellular or inside multinucleated giant cells.

The majority of *Coccidioides* infections are mild or even asymptomatic (2). However, inhalation of arthroconidia can cause more severe infections, and in the areas of endemicity, coccidioidomycosis accounts for a large percentage of cases of pneumonia (3). Whether symptomatic or not, most immunologically intact people recover spontaneously from the primary infection, but ~5% of infections spread to extrathoracic sites, and these do not resolve spontaneously. Immunosuppression (4, 5), including the immunological changes that occur in the third trimester of pregnancy (6), is a risk factor for dissemination of infection, but disseminated infections can occur in previously healthy individuals with no known immune defects (7). Little is known about the risk factors for dissemination in normal hosts, but genetic factors are important, since African Americans and Filipinos are 5 to 10 times more likely than Caucasians to develop disseminated infections (8–10). Many studies that linked race/ethnicity with outcome of infection were done on military bases in the San Joaquin Valley of California, where other confounders, such as nutritional status, living conditions, prior exposure, etc., were not significant variables (11–13).

People with disseminated coccidioidomycosis make high titers of antibody to fungal antigens, whereas patients with self-limited infections generate only low titers of antibodies, and they develop long-lasting delayed-type hypersensitivity (DTH) to fungal antigens (14). This suggests that dissemination is associated with a Th2 immune response and self-limited infections with Th1 responses. In further support of genetic factors being important in determining the immune response to infection and therefore susceptibility, we discovered that inbred mice also vary greatly in their susceptibility to coccidioidomycosis (15). Susceptible mice make more interleukin 4 (IL-4) and IL-10 (16, 17), and IL-10-deficient C57BL/6 (B6) mice are more resistant to infection than B6 mice (18).

The genes responsible for resistance in mice have not been identified, but one locus mapped to chromosome 6, not far from the cluster of *Clec* genes that includes *Clec7A* (which encodes Dectin-1) and *Clec4N* (also known as *Clec6A*; encodes Dectin-2). Both of those C-type lectin receptors (CLR) are transmembrane proteins expressed on myeloid cells (19). Dectin-1 is alternatively spliced by humans and mice (20, 21), which results in expression of two functional membrane-bound proteins of different lengths that appear to have different functional characteristics *vis-à-vis* binding to *Candida albicans* (22). In a set of BXD recombinant inbred mice, we found an association between the expression of full-length Dectin-1 and resistance to coccidioidomycosis (20). Expression of Dectin-1 on macrophages is regulated by a number of cytokines, and we found that genetically resistant DBA/2 mice

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express much more Dectin-1 in their infected lungs than do two genetically susceptible mouse strains (23). However, even this reduced level of Dectin-1 expression is somewhat protective, because Dectin-1 knockout (KO) mice are even more susceptible than B6 mice.

A great deal has been learned in the last 2 decades about how innate immunity influences acquired immunity to microbes (24). The general strategy for recognizing invading microbes is the deployment of a limited number of receptors (which are conserved across broad taxa) that recognize structures that are unique to the microbes (25). The host molecules that recognize components of microbes are called pattern recognition receptors (PRRs) (26). Ligation of a PRR leads to transcriptional changes in the cells. One type of PRR is CLR. They form a large family of proteins that are highly conserved in vertebrates (27). In recent years, CLR have been established as part of the antifungal innate immune system (28, 29). There is experimental evidence in mice that CLR are important for resistance to opportunistic fungal pathogens, such as *Candida albicans* and *Pneumocystis carinii* (30, 31). We recently showed that Dectin-1 is required for resistance to *Coccidioides immitis* in mice, and so far this is the only example of Dectin-1 playing a role in resistance to a primary pathogenic fungus (23). A mutation in human *Clec7A* that deletes the carbohydrate recognition domain on Dectin-1 predisposes to a mild form of vulvovaginal candidiasis and to onychomycosis, demonstrating that the gene plays a nonredundant role in resistance to mucocutaneous fungal infections in humans (32). A mutation in *Card9* (caspase recruitment domain-containing protein 9), which is part of the signaling cascade from Dectin-1, causes a more severe form of mucocutaneous candidiasis, invasive candidiasis, and dermatophytosis (33) (34). The mutation in *Card9* affects NF-κB activation and compromises the Th-17 immune response to fungi such as *C. albicans* (35).

The composition of spherule and endospore cell walls is not fully defined, but they contain several polysaccharides, including chitin, β-1,3-glucan (the ligand for Dectin-1), 3-*O*-methyl-mannose, and mannans (36, 37). Dectin-2 binds high-mannose residues (38), and the mannose receptor (MR) binds terminal mannose residues (39). The MR is an endocytic receptor that mediates clearance of endogenous glycoproteins (40), but it also binds to fungi, including *C. albicans* (41) and *Pneumocystis carinii* (42). The MR may not be an opsonic receptor, but it appears to play a role in cell attachment and in cell signaling, though probably indirectly (43). Dectin-2 also binds *C. albicans*, and it is both an opsonic and a signaling receptor, partnering with the FcRγ chain, which has an intracellular immunoreceptor tyrosine-based activation motif (ITAM) (44). Dectin-2 has been shown to be required for resistance to *C. albicans* in mice, whereas the MR is not required for resistance to that fungus (41) or to *P. carinii* (42). Because these two CLR bind to mannose residues that are likely to be part of the spherule cell wall structure, we investigated the roles of these two genes in resistance to coccidioidomycosis.

## MATERIALS AND METHODS

**Mice.** Mice with a targeted mutation in *Clec4n* (Dectin-2 KO) on a C57BL/6 (B6) background were described previously (45). *Mrc1*$^{-/-}$ (MR KO) mice on a B6 background were the kind gift of Michel Nussenzweig (Rockefeller University) (40). *Clec7a* knockout mice in a B6 background were originally created by Gordon D. Brown (46) and were kindly provided by Stuart Levitz (University of Massachusetts Medical School). To create homozygous Dectin-2/MR mice, we bred F2 mice, screened them by PCR to identify mice that were homozygous for both mutations, and then crossed them to make the doubly deficient strains. B6 female mice were purchased from Jackson Laboratory (Bar Harbor, ME).

**Infection.** Mice were infected intranasally as previously described (23). All experiments were carried out by procedures authorized by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System.

**Fungus.** *C. immitis* strain RS was used for all *in vivo* infections. For *in vitro* cell activation assays, we used formalin-killed spherules (FKS) that were harvested 96 h after culturing of arthroconidia in Converse medium (a generous gift from John Galgiani, University of Arizona). To remove mannose residues without destroying β-glucan, we boiled the FKS in 10 N NaOH for 30 min to remove neutral sugars other than β-1,3- and β-1,6-glucan (A-FKS) and then washed the A-FKS 5 times with pyrogen-free phosphate-buffered saline (PBS) (47).

**Mouse peritoneal macrophage activation.** Inflammatory macrophages were elicited in the peritoneal cavity by injection of sodium periodate and were harvested using ice-cold PBS 4 days after injection (48). We cultured $1 \times 10^6$ cells/ml in high-glucose Dulbecco's modified Eagle medium (DMEM) with 10% endotoxin-free fetal calf serum (FCS) in a 48-well tissue culture plate and activated them overnight as described previously (49). We used the synthetic Toll-like receptor 2 (TLR2) agonist Pam3CysSerLys4 (Pam3CSK4) as the control for activation.

**Mouse bone marrow-derived dendritic cells (BMDC).** We cultured bone marrow cells obtained from mouse femurs and tibias in RPMI with 10% fetal calf serum in the presence of 5 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (BD Bioscience) as described by Lutz et al. (50). New medium was added on day 3, and the nonadherent cells were harvested on day 6. Dendritic cells (DCs) were positively selected for using anti-CD11c magnetic beads (Miltenyi, Auburn, CA) according to the manufacturer's protocol. The BMDC were plated at a density of $1 \times 10^6$ cells/ml in RPMI with 10% endotoxin-free FCS, and we incubated them overnight with various numbers of FKS in 96-well flat-bottom tissue culture plates. The highest ratio of FKS to cells was 1:1. We used Pam3CSK4 as the positive control for activation.

**Mouse bronchoalveolar lavage.** Mice were euthanized by asphyxia in a $CO_2$ atmosphere. Tracheae were exposed and intubated with a 16-gauge blunt-end needle. Four successive installations into the trachea of 0.2 ml of sterile saline were aspirated as completely as possible using a 1.0-ml syringe. Cells in the aspirates were sedimented by centrifugation at 400 × *g* for 10 min at 4°C. The lung lavage supernatant was then centrifuged through a 0.45-μm centrifuge tube filter (Spin-X; Costar) to remove any remaining fungal cells. The bronchoalveolar lavage (BAL) fluid was assayed immediately for cytokines or stored at −20°C until used.

**Adherence assay.** Adherence of FKS to cells was measured as previously described with one modification (23). Briefly, purified BMDC were allowed to adhere to 16-well glass Lab-Tek slides (Thermo, Fisher, Waltham, MA), and then various ratios of FKS or A-FKS were added to the wells. After 6 h, the nonadherent spherules were washed off and the slides were stained with Wright's stain. For each set of conditions we had 2 wells, and in each well we identified several hundred morphologically intact DCs present in contiguous fields as viewed with an oil emersion lens at magnification ×50, and the percentage of cells with adherent or ingested FKS was calculated.

**Cytokine assays.** Mouse cytokines in BAL fluid were measured using enzyme-linked immunosorbent assay (ELISA) kits as described in the manufacturer's protocols. The ELISA kits for tumor necrosis factor alpha (TNF-α), IL-6, IL-4, IL-10, IL-17, IL-23, IL-12p70, transforming growth factor β (TGF-β), and gamma interferon (IFN-γ) were purchased from eBioscience (San Diego, CA). The assay kits for IL-1β, IL-12p40, and GM-CSF were from BD Biosciences; macrophage inflammatory protein 2 (MIP-2) and IL-22 assay kits were from R&D. The FlowCytomix multiplex Th1/Th2/Th17/Th22 13-plex cytokine assay (eBioscience) was used in the experiments with isolated macrophages and BMDC. After we stim-

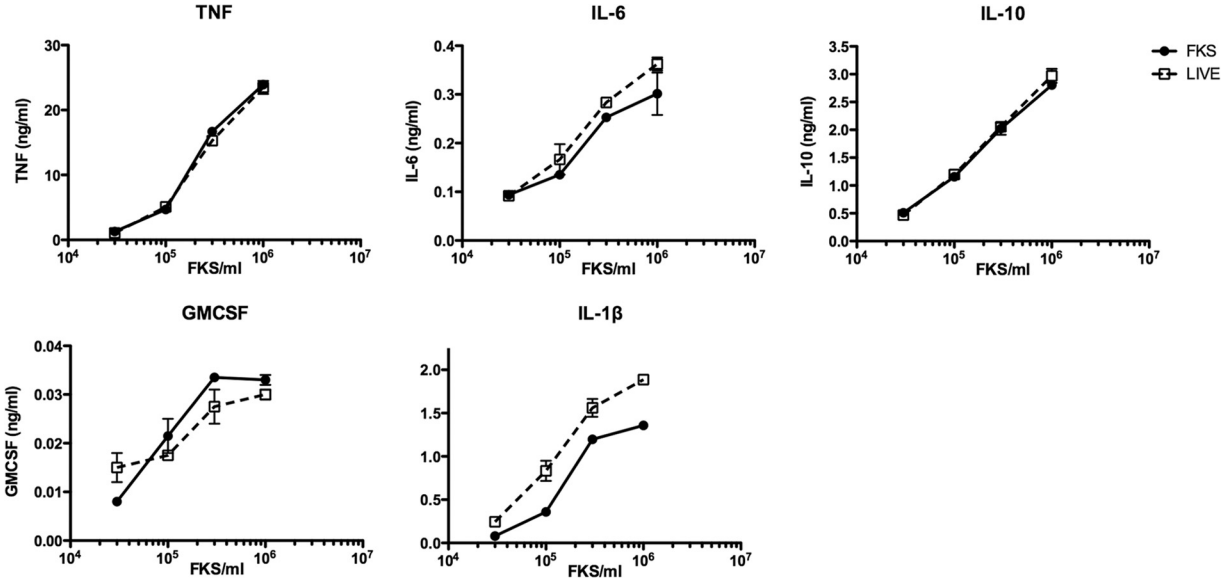


**FIG 1** Comparison of live spherules and FKS. Spherules were incubated overnight with BMDC from B6 mice, and the supernatants were removed to measure the concentration of cytokines in the supernatant. Because of the biohazard of live spherules, the supernatants were sterile filtered through 0.45-μm filters. Supernatants from live spherules and FKS were handled the same way. Due to loss of sample volume from filtering, we could measure only 5 cytokines. There were no differences between the responses of BMDC to live and killed spherules.

ulated BMDC with live 92-h spherules, we filtered the supernatants through a 0.45-μm filter to remove organisms before the supernatants were assayed for cytokines by ELISA as described above.

**Statistics.** Means were compared using an unpaired *t* test (GraphPad Prism5; GraphPad, San Diego, CA).

## RESULTS

We previously showed that live spherules and FKS are equivalent in their abilities to stimulate inflammatory macrophages to secrete proinflammatory cytokines and chemokines (49). Since we had not established that live and formalin-killed spherules were equivalent for activation of BMDC, we compared the abilities of FKS and freshly made live spherules to stimulate cytokine production by B6 BMDC; there were no differences between their stimulatory activities as measured by cytokine secretion (Fig. 1).

Because live spherules are a biohazard, we used FKS in all subsequent *in vitro* experiments with isolated myeloid cells. We first incubated increasing numbers of FKS with elicited peritoneal macrophages from MR KO and control B6 mice, and we measured secreted TNF-α, IL-6, and MIP-2 in the supernatant. The dose-response curves are shown in Fig. 2A. B6 macrophages secreted significantly more of those three cytokines in response to FKS, but they made equivalent responses to Pam3CSK4 (not shown). We then compared the responses of BMDC from B6 and MR KO mice (Fig. 2B). B6 BMDC made ~5-fold more TNF-α, IL-6, IL-10, GM-CSF, and IL-1β than did $MR^{-/-}$ BMDC at the maximal stimulatory ratios of FKS to BMDC. We did not detect measurable amounts of IL-12p70, IL-17A, IL-23, or TGF-β in the supernatants of BMDC from either strain of mice.

There is a great deal of controversy about whether the MR is involved directly in signaling or phagocytosis, but it does bind ligands, and it is an endocytic receptor (43) (51). Since FKS are particulate and cannot secrete antigens because they are killed, we reasoned that direct contact between FKS and BMDC would be necessary in order to stimulate signaling. The MR has been shown to be both positively and negatively involved in phagocytosis of different mycobacteria and fungi (52, 53), so we compared the abilities of B6 and MR KO BMDC to bind to FKS. As shown in Fig. 2C, only half as many MR KO BMDC were associated with FKS than was the case for control B6 BMDC, implying that the MR was required for attachment of spherules to DCs. In contrast, MR KO BMDC were not impaired in their adherence to A-FKS. This result was anticipated because hot alkali treatment removes mannose residues from fungi but does not remove β-glucan from FKS, and MR KO cells still express Dectin-1, the opsonic receptor for β-glucan (23). Others have shown that MR and Dectin-1 collaborate in the phagocytosis of heat-killed *Saccharomyces cerevisiae* by murine macrophages (54). Combined with our previous results concerning Dectin-1 and BMDC (23), results show that both receptors are required for maximal interaction of BMDC with spherules.

We then infected MR KO mice and B6 controls, and we collected BAL fluid at the time of necropsy (Fig. 3). MR KO mice had significantly higher concentrations of the Th17 cytokines IL-17A and IL-23, cytokines that are protective against fungal infections. MR KO mice had lower levels of IL-6, and both groups of mice had equally low levels of the potentially protective cytokines IFN-γ and TNF-α. The IL-6 result is consistent with the *in vitro* finding that $MR^{-/-}$ macrophages and BMDC made less IL-6 than control cells in response to FKS, but MR KO mice still had nanogram amounts of IL-6 in the BAL fluid, perhaps because IL-6 is also secreted by a variety of nonleukocytes, such as endothelial, fibroblastic, and epithelial cells, which could be involved in the *in vivo* IL-6 response (55). IL-6 is important for the development of Th17 $CD4^+$ T cells (56), so the *in vivo* production was apparently adequate to direct T cell development.

Despite the evidence that MR was involved in recognition of FKS by BMDC and in activation of both elicited peritoneal macrophages and BMDC, MR KO mice were not more susceptible to infection than B6 controls, as measured by numbers of CFU re-

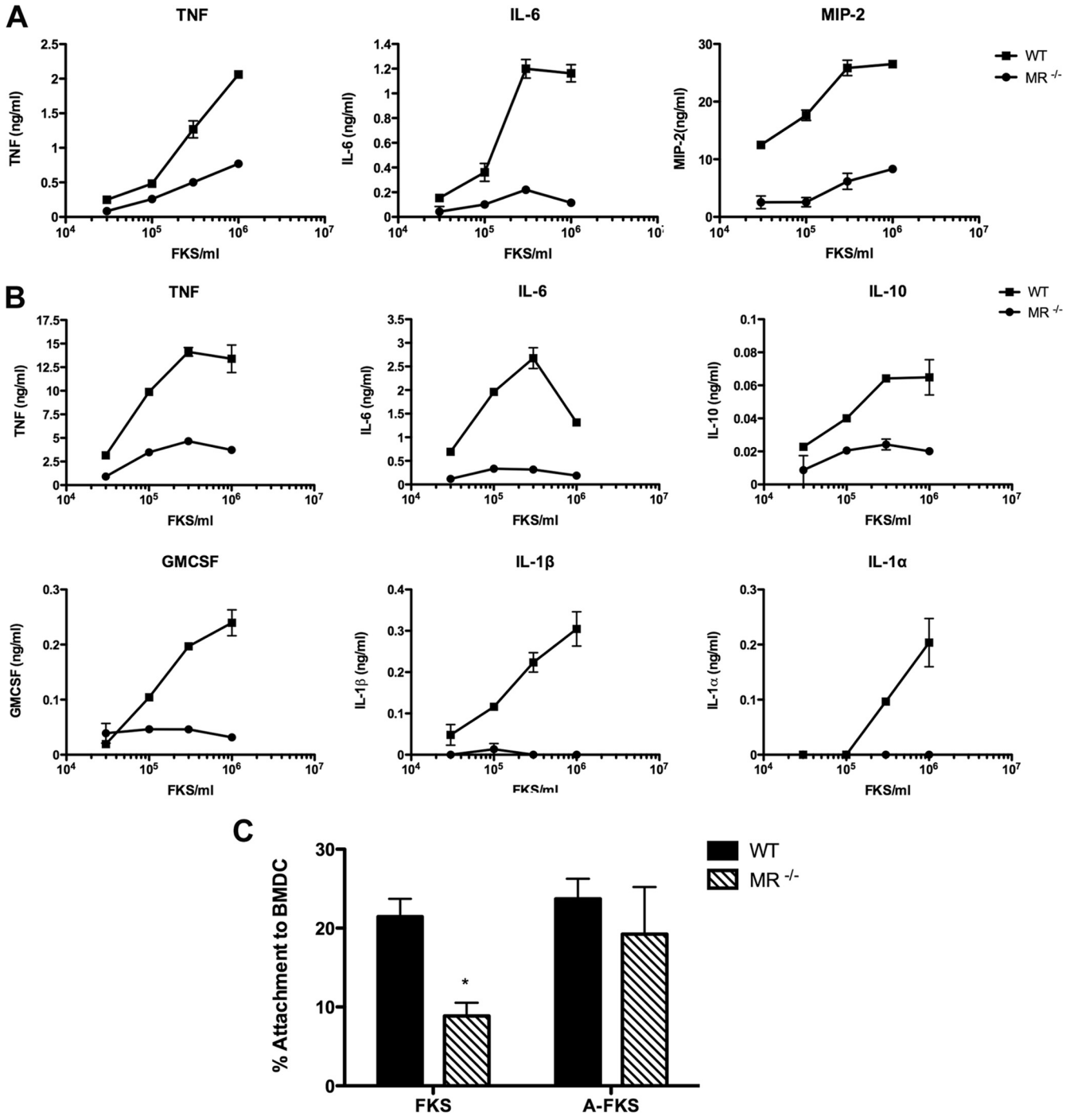


**FIG 2** Comparison of the responses to spherules by cultured myeloid cells from B6 and MR KO mice. (A) Dose-response curves for elicited macrophages in response to increasing numbers of FKS, determined in duplicate. WT (B6) cells are represented by squares, MR KO cells by circles. (B) Dose-response curves for WT (B6) and MR KO BMDC in response to increasing numbers of FKS. (C) The attachment of FKS to MR KO BMDC (striped bars) was only half that of B6 BMDC (black bars) (*, $P < 0.05$). In contrast, A-FKS attached equally well to BMDC from the two mouse strains.

covered from lungs or spleens 14 days after infection (Fig. 4). The amount of dissemination to spleens was slightly less in the MR KO mice, suggesting they are more resistant to disseminated infection, but that difference was not statistically significant ($P = 0.08$). Thus, the impaired *in vitro* responses of $MR^{-/-}$ myeloid cells to FKS did not predict how the mutant mice would respond to actual infection.

We next examined the role of Dectin-2, comparing the Dectin-2 KO mice to B6 controls. When we compared the responses of elicited peritoneal macrophages to FKS, we found that the Dectin-2 KO macrophages made almost no measureable TNF-α, IL-6, or MIP-2 (Fig. 5A); the responses of the two cell types to Pam3CSK4 were not significantly different (not shown). We also compared the amounts of cytokines made by B6 (control) and

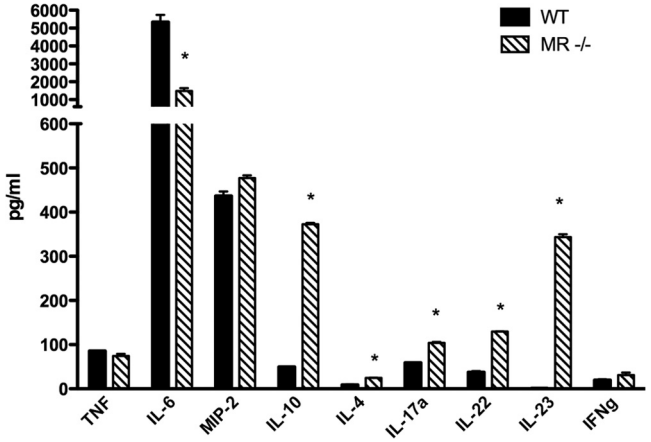


**FIG 3** Cytokine concentrations in lung lavage fluids from infected MR KO mice. Each bar is the mean from the pooled BAL fluid from 4 mice. There were no significant differences in the MIP-2, IFN-γ, or TNF-α levels. MR-KO mice had higher levels of IL-10, IL-4, IL-17α, IL-22, and IL-23, though the concentration of IL-4 was low in both groups. B6 mice made more IL-6. *, $P < 0.05$.

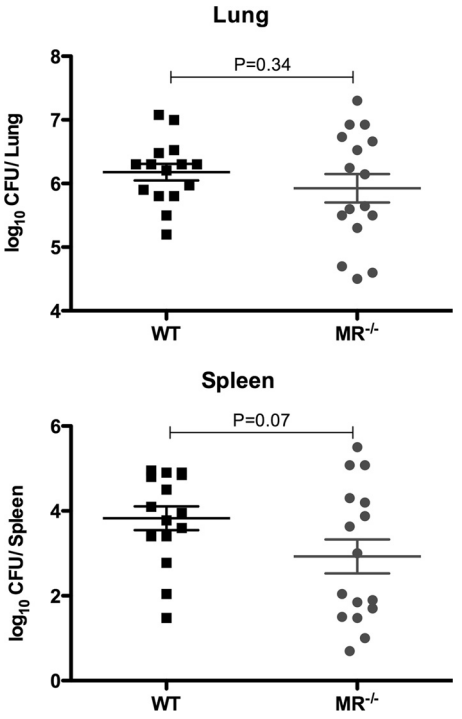


**FIG 4** Comparisons of CFU in lungs and spleens of B6 and MR KO mice 14 days postinfection (p.i.). This figure combines results from two independent experiments. Each symbol represents one mouse, and the geometric means ± 1 SD are shown by the horizontal lines. Uninfected mice were excluded from analysis.

Dectin-$2^{-/-}$ BMDC in response to FKS, and as shown in Fig. 5B, there was no difference in the amounts of TNF-α, IL-6, IL-1β, and GM-CSF made by the BMDC from the two strains of mice, although the Dectin-2 mutant cells produced only half as much IL-10.

We then infected Dectin-2 KO mice intranasally, and 14 days later we carried out quantitative cultures of their lungs and spleens. As shown in Fig. 6A, there was no difference between numbers of CFU recovered from the tissues of Dectin-2 KO mice and controls. We then considered the possibility that there was redundancy in the two CLR that recognize complex mannans, so we bred double MR/Dectin-2 KO mice and infected them. Again, there was no difference between numbers of CFU recovered from the lungs and spleens of the MR/Dectin-2 KO and B6 controls (Fig. 6B). Note that there is experiment-to-experiment variability in the severity of the lung infections in the controls, in part due to variability in the number of organisms that are inhaled into the lung, which is why we always include a B6 control when we test a mutant mouse strain and only make intraexperiment comparisons.

We also measured cytokines in the BAL fluid from infected B6, Dectin-2 KO, and MR/Dectin-2 KO mice (Fig. 7). There were no significant differences in the concentrations of any cytokines except for IL-1β, which was higher in the Dectin-2 KO lungs. Both Dectin-2 KO mice and the double mutants had more than twice as much IL-10 than the B6 controls, even though Dectin-2 BMDC made less IL-10 *in vitro*. We did not determine the cellular source of the IL-10 in the lungs.

## DISCUSSION

The principal finding in this study is that deficiency of Dectin-2 or the MR, singly or in combination, does not affect the susceptibility of B6 mice to *C. immitis* infection. This result adds *C. immitis* to the list of fungi, including *C. albicans* (41) and *P. carinii* (42), which bind to the MR but are not more virulent in MR KO mice. An exception is *Cryptococcus neoformans*. The MR recognizes cryptococcal mannoproteins and participates in antigen presentation to T cells (57), and Dan et al. found that MR KO mice are more susceptible to *C. neoformans* infection (58). It is not understood why mutation of *Mrc1* is not detrimental in most fungal infections, but the MR is a marker for M2 macrophages (59), and M2 macrophages generally do not have potent antimicrobial activity, at least *in vitro* (60). Engagement of the macrophage MR has been shown to trigger anti-inflammatory responses, and this could be detrimental to the immune response to *C. immitis* infection (61, 62).

There have been few studies of the role of human MR in response to *Coccidioides*. Ampel et al. (63) assessed the response of peripheral blood mononuclear cells (PBMC) to T27K, a soluble extract of spherules. Mannose is the principal monosaccharide (1,151 nM/mg) in T27K. T27K stimulates human PBMC to secrete IL-2 and IFN-γ, and this response is blocked by soluble mannans. This group went on to show that immature heat-killed spherules are ingested by blood-derived DCs and their uptake is blocked by mannan (64). This was interpreted as evidence that the MR mediated those responses, although there are several mannose-binding receptors on PBMC. Whether the human MR plays a role in resistance to human infection is unknown.

Few studies of the role of Dectin-2 in resistance to fungal infections have been done. Saijo et al. showed that Dectin-2 was important for resistance to systemic infection with *C. albicans* but not *C. neoformans* (45). Dectin-2 can recognize several fungi that typically cause superficial skin and corneal infections (44, 65, 66), but no studies have been done to determine whether Dectin-2 is necessary for resistance to those fungi.

We found that both MR KO and Dectin-2 KO mice had even more IL-10 than control B6 mice in the BAL fluid from infected

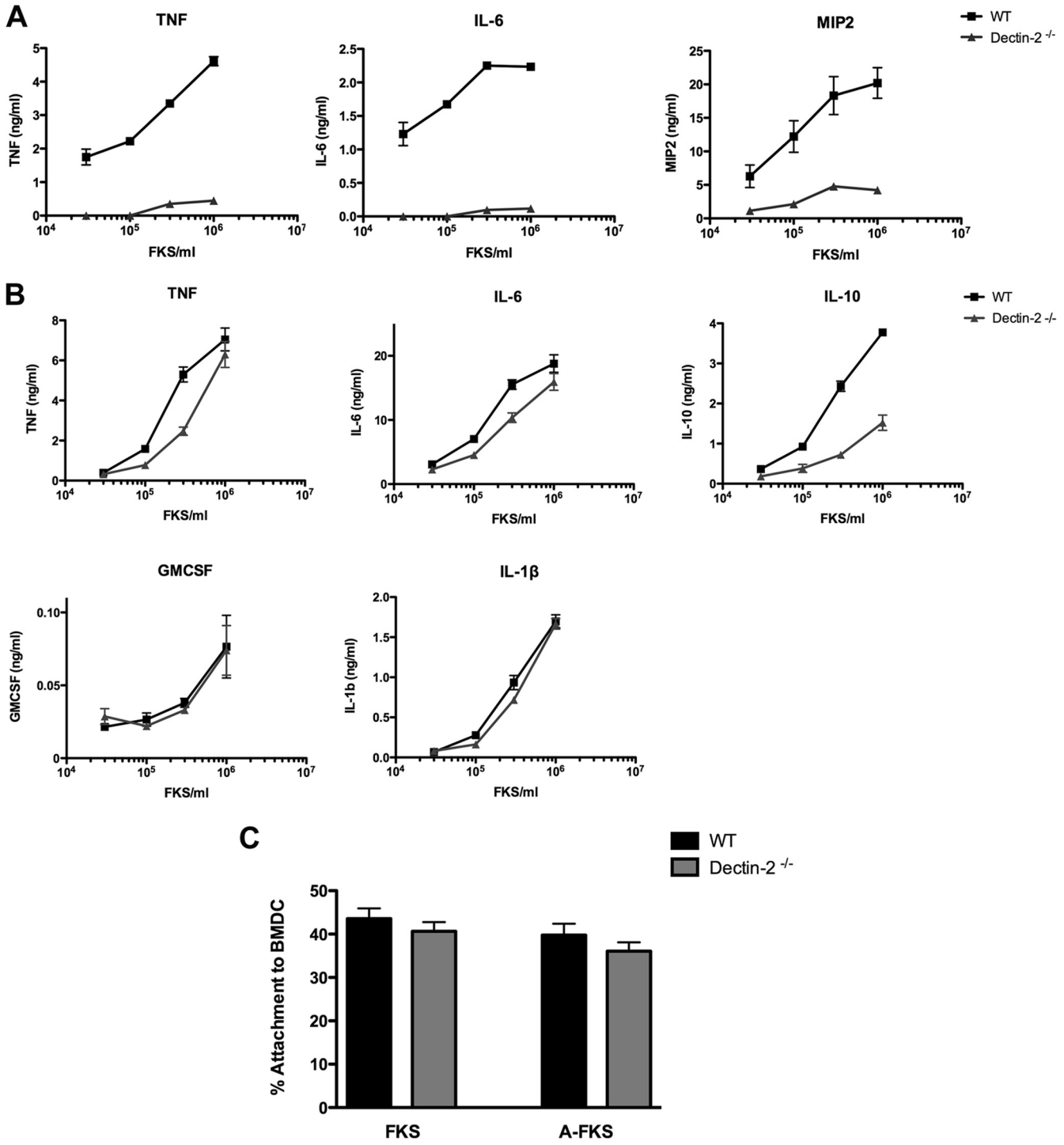


**FIG 5** Dectin-2 and FKS/A-FKS. B6 (WT) mice are represented by square symbols and Dectin-2 KO mice by round symbols. FKS-stimulated cells are solid lines; A-FKS-stimulated cells are dashed lines. (A) Dectin-2 KO macrophages did not make TNF-α, IL-6, or MIP-2 in response to either FKS or A-FKS. They responded equally to Pam3CSK4 (not shown). (B) Secretion of TNF-α, IL-6, IL-1β, and GM-CSF by BMDC was not dependent on Dectin-2, but IL-10 secretion was reduced ~50% in the absence of Dectin-2. (C) Dectin-2 was not required for attachment of FKS and A-FKS to BMDC.

lungs, but the mutant mice were not more susceptible to infection (Fig. 4 and 6). This was a surprising result, because B6 mice make more IL-10 than resistant DBA/2 mice (67), and high levels of IL-10 are detrimental in this infection (17). One possible explanation is that the mutant mice also made more of some potentially protective cytokines, such as IFN-γ, GM-CSF, and IL-17 and IL-23. We do not know how these protective cytokines mediate resistance to *C. immitis*, nor do we know the exact relationship between the concentrations of these cytokines and resistance to infection, but we do know that complete absence of those cyto-

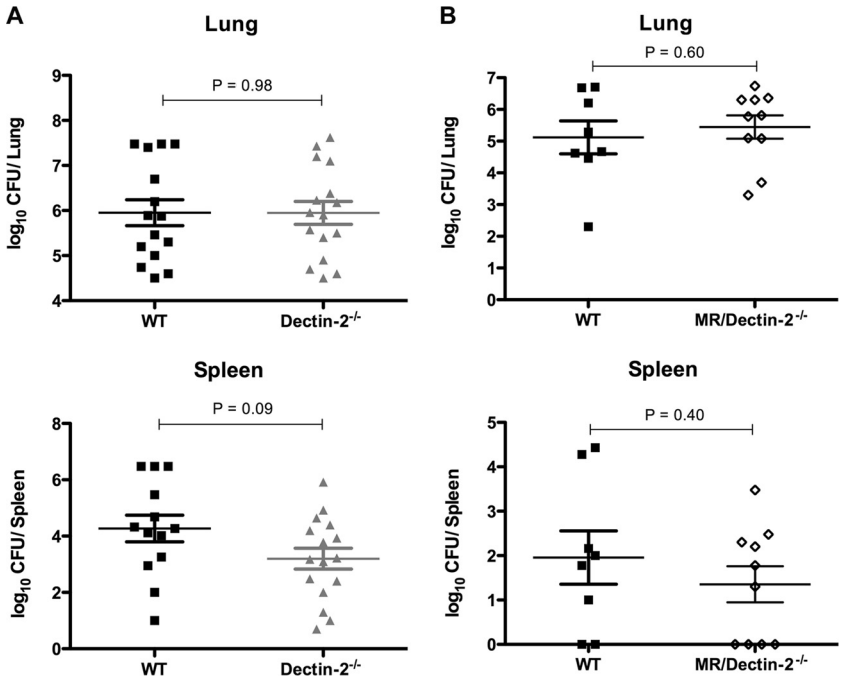


**FIG 6** CFU in lungs and spleens of B6 and Dectin-2 mice and Dectin-2/MR KO mice 14 days p.i. Each point represents a single mouse, and the horizontal lines are the geometric means ± 1 SD. Uninfected mice were excluded from analysis. (A) B6 versus Dectin-2 KO mice had nearly identical numbers of CFU in their lungs and slightly fewer CFU in their spleens, but this was not statistically significant. This figure shows the combined results of two independent experiments. The WT results were previously published (23). (B) There was no significant difference between numbers of CFU recovered from B6 versus Dectin-2/MR double KO mice.

kines or their receptors predisposes mice and humans to severe coccidioidomycosis (16, 68–70).

It was of interest to know which C-type lectins are involved with the recognition of the fungus and with stimulation of cytokine secretion by myeloid cells, since those cells are important both in the innate immune response and in shaping the acquired immune response. Therefore, we measured cytokines made by elicited peritoneal macrophages and BMDC exposed to FKS. $MR^{-/-}$ BMDC and elicited peritoneal macrophages made less of all the proinflammatory cytokines that we measured (Fig. 2). Heinsbroek et al. also found that $MR^{-/-}$ elicited peritoneal macrophages made less TNF-α in response to *C. albicans*, although they did not find that the MR was involved in phagocytosis (71). Yamamoto et al. used antisense oligonucleotides against *Mrc1* to inhibit expression of the MR by elicited peritoneal macrophages, and that decreased their synthesis of IL-1β, IL-6, and GM-CSF mRNA, but there was no reduction in MIP-2 message (72). In substantial agreement with these other studies, we found that FKS-stimulated $MR^{-/-}$ macrophages secreted less IL-6, TNF-α, and MIP-2 (Fig. 2A), but there was no correlation between the *in vitro* findings and the cytokine levels in the lungs. As Murray and Wynn pointed out, macrophage responses are very dependent on their activation state, and that can be very complex *in vivo* and different from *in vitro* activation (73). Clearly the cellular milieu in an infected lung is much more complex and dynamic than can be approximated *in vitro* with purified cell types. However, our experimental design did not exclude the possibility that decreased cytokine production by BMDC and macrophages was the indirect result of decreased adherence of FKS to myeloid cells. This has to be considered because others have shown that the MR by itself is not a signaling receptor and the MR lacks signaling motifs in its cytoplasmic domain, so it must participate with other receptors to initiate a signaling cascade (74).

We found that Dectin-$2^{-/-}$ macrophages were more impaired in their responses to FKS than were Dectin-$2^{-/-}$ BMDC (Fig. 5). We do not know why the two cell types had different requirements for Dectin-2, but others have shown that mouse bone marrow-

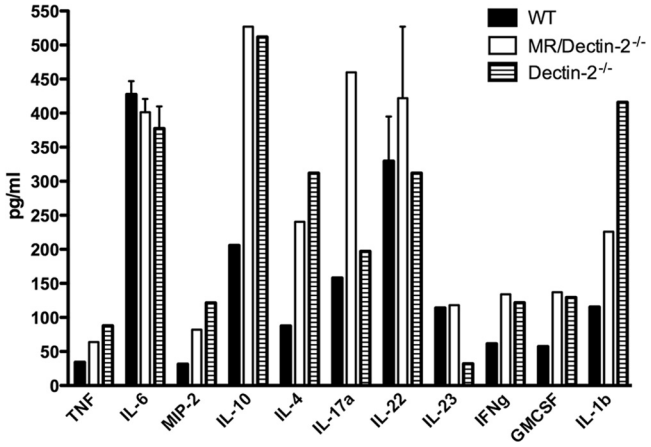


**FIG 7** Cytokine concentrations in lavage fluid from infected lungs of B6, Dectin-2 KO, and MR/Dectin-2 KO mice. We pooled equal amounts of lavage fluid from 4 mice in each group and carried out the assays in triplicate. Both KO strains had more IL-10 and IL-4 than B6 controls. Dectin-2 KO mice had more IL-1β than the control. MR/Dectin-2 KO mice had more IL-17A than the other 2 strains. None of these differences reached statistical significance ($P > 0.05$, analysis of variance [ANOVA]).

derived macrophages and BMDC react differently to Dectin-1 agonists because macrophages and BCs are reprogrammed by cytokines used as *in vitro* growth factors (75). Municio et al. recently showed that human macrophages and DCs responded differently to zymosan and β-glucan depending on whether or not M-CSF was used to mature the cells or if TLR ligands or IFN-γ were added to the reaction mixture (76). In our *in vitro* assays with BMDC, we did not detect IL-12p70 or IFN-γ, perhaps because we were studying genetically susceptible B6 mice, which do not generate Th1 immune responses to this infection (20, 77).

For both CLR mutants, there was no apparent correlation between the *in vitro* responses of macrophages and lung cytokine levels. For instance, deletion of the MR resulted in decreased IL-10 production by isolated BMDC (Fig. 2), while there was nearly 10 times more IL-10 in their infected lungs (Fig. 3). The explanation for this apparent discrepancy is not known, but IL-10 can be produced by several other cell types, including macrophages, regulatory T ($T_{reg}$) cells, and NK cells (reviewed in reference 78), and we did not investigate the source of IL-10 in infected lungs. Another possible explanation is that we used elicited peritoneal macrophages instead of alveolar macrophages in our assays. However, resting peritoneal macrophages are distinct from elicited peritoneal macrophages, which are inflammatory macrophages that are largely Ly6c positive (79, 80), and 75% of the macrophages in the BAL fluid from infected B6 lungs were CD11b/Ly6c high (not shown).

Dectin-1 and Dectin-2 are C-type lectin receptors that are expressed on DCs and macrophages (27, 81). Although they have different ligands and Dectin-2 combines with the FcRγ chain to mediate intracellular signaling (44), upon ligation both are phosphorylated and bind Syk, which starts an intracellular signaling cascade through CARD9-Bcl10-Malt1 that leads to activation of NF-κB (82). The reason for the importance of Dectin-1 but not Dectin-2 in *C. immitis* infection is not known but probably reflects differences in the carbohydrates that they recognize. This is supported by the fact that Dectin-$1^{-/-}$ BMDC have impaired recognition of FKS (23), whereas in this study we showed that Dectin-$2^{-/-}$ BMDC attached normally to FKS. Although the exact structure of the ligands for Dectin-2 has not been determined, Dectin-2 is a low-affinity mannose binder, and on a glycan array it has the highest affinity for $Man_9GlcNac_2$. By competition studies, the Dectin-2 ligand is distinct from the MR and SIGNR-1 ligands (24). Zymosan, which can activate both Dectin-1 and Dectin-2, increases the transcription and secretion of IL-12p70, IL-23, IL-6, and IL-1β by BMDC, which in turn promotes the development of Th1 and Th17 immune responses. Robinson et al. found that BMDC from B6 mice required only 1 of the two Dectin receptors in order to make TNF-α and IL-10 in response to zymosan (83), implying that the function of the two receptors was redundant if a fungal particle is recognized by both receptors. Mutation of either receptor makes mice more susceptible to systemic *Candida albicans* infection, though this may depend on the strain of *C. albicans* used to infect the mice (31, 84, 85).

There are limitations of this study. We studied only one type of macrophage, and macrophages are heterogeneous in terms of their receptors and the way they respond to microbes (86). It may be impossible to recreate *in vitro* the complex phenotypes of macrophages that are present during an infection (86). It is also possible the FKS is not sufficiently representative of the fungal particles that are present in infection. *In vivo* spherules have an outer membrane made up of membranous glycoprotein (SOWgp) (87) that can be lost during *in vitro* cultivation, and SOWgp could interact with other receptors or prevent recognition by CLR of mannose ligands in the cell wall. In addition, we used FKS that were too large to be ingested and so probably did not interact with intracellular TLRs that influence the way macrophages and DCs respond to zymosan (76). Since a decrease in cytokine production by Dectin-$1^{-/-}$ macrophages and BMDC *in vivo* does predict greater susceptibility to infection (23), we can only conclude that *in vitro* results may not predict susceptibility, which needs to be determined *in vivo*. Finally, all these studies were done in mice on a B6 genetic background, and since these mice are already genetically susceptible to *C. immitis* infection (15), it may be difficult to discover deleterious mutations.

In summary, we have shown that deficiencies of MR and Dectin-2, either singly or in combination, affect cellular responses to FKS *in vitro* but do not make C57BL/6 mice more susceptible to pulmonary coccidioidomycosis.

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